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(54) Title: A COMBINATION OF ANTI-erbB-2 MONOCLONAL ANTIBODIES AND METHOD OF USING

(57) Abstract

The present invention relates to a combination of at least two monolconal antibodies which are capable of preventing and treating human malignancies wherein the malignant cells overexpress gp185^{erbB-2}. The monoclonal antibodies of the combination recognize different epitopes of the gp185 expression product of erbB-2, therefore, the antibodies do not cross react with each other. Preferably, the combination decreases the expression product of the erbB-2 gene. In another embodiment, the combination does not essentially increase tyrosine phosphorylation of the gp185 expression product.

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A COMBINATION OF ANTI-erbB-2 MONOCLONAL ANTIBODIES AND METHOD OF USING

The present invention relates to a combination of monoclonal antibodies capable of preventing and treating tumors. More specifically, the monoclonal antibodies are single chain monoclonal antibodies which are capable of treating and preventing tumors.

Amplification and/or overexpression of the erbB-2 gene (also called HER-2 or neu) results in overexpression of erbB-2 mRNA and proteins and has been demonstrated in 20-30% of adenocarcinomas of the breast (1-5), ovary (3), lung (6) and stomach (7). Two lines of evidence implicate erbB-2 overexpression in the pathogenesis of human neoplasia. First, overexpression has been linked with poor prognosis in breast (8-11), ovarian (12), stomach (13), and lung cancer (14), indicating that overexpression alters the cancer cell. Second, artificial overexpression of erbB-2 induces a transformed phenotype in NIH/3T3 fibroblasts (15, 16) as well as in mammary epithelial cells (17) suggesting that overexpression can contribute directly to the development of the malignant phenotype.

Due to the extensive homology between $gp185^{erbB-2}$ and the epidermal growth factor receptor (EGFR), it is widely assumed that their activation might proceed through similar mechanisms. One such mechanism involves receptor dimerization/oligomerization which is thought to be an important step in the activation of the EGFR intrinsic tyrosine kinase function (18, 19). Interfering with receptor-receptor interactions has been evaluated as a potential therapeutic approach to treatment of cancers with erbB-2 overexpression. Previous studies have evaluated the use of single monoclonal antibodies directed against erbB-2

(20) and the related Epidermal Growth Factor Receptor (EGFR) protein (21) as potential therapeutic agents for the treatment of cancer.

Use of single monoclonal antibodies directed against erbB-2 which have been evaluated as potential therapeutic agents have resulted in increased $gp185^{erbB-2}$ autophosphorylation, resulting from increases in the activity of tyrosine kinase. Single antibody agents have shown promise as potential anticancer therapies (20, 27).

SUMMARY OF THE INVENTION

One object of Applicant's invention relates to a combination of at least two monoclonal antibodies capable of treating or preventing human malignancies wherein the malignant cells overexpress $gp185^{erbB-2}$. The combination comprises at least a first and second antibody each of which recognizes the gp185 extracellular domain of erbB-2. The activity demonstrated by the combination antibody treatment has shown greater activity than expected by the sum of the individual antibodies at the same overall antibody concentration.

Another object of the present invention provides for the use of monoclonal antibodies which are single chain monoclonal antibodies. The single chain antibodies can be used to form a bispecific antibody.

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1A. Specificity of monoclonal antibodies #21 and #23. Subconfluent SK-Br-3 monolayers were metabolically labeled with 35S-Cys (spec. act. 1000 Ci/mmol). Total cell proteins were immunoprecipitated with 10 μg of the indicated antibodies. The immune complexes were recovered by Protein G Agarose (Genex, Gaithersburg, MD) and analyzed by SDS-PAGE

on an 8-16% Tris-Glycine gel. The gel was exposed to film at -70°C overnight with an intensifying screen.

Figure 1B. $gp185^{erbB-2}$ overexpression in the gastric cell line N87 and a tumor from N87 mouse xenografts compared to high and low $gp185^{erbB-2}$ overexpressers. Cells or tumor were lysed in sample buffer which contained 0.125 M Tris-HCl, 4% SDS, 0.002% bromophenol blue, and 15% glycerol. 5% β-mercaptoethanol was added after the protein concentration was determined. Samples (10 μg total protein) were boiled for 3 min, fractionated by SDS-PAGE on 8-16% Tris-Glycine gel and transferred to nitrocellulose. Detection of $gp185^{erbB-2}$ was performed with a monoclonal antibody to the c-terminal portion of the protein.

Figure 1C. Southern blot analysis of the erbB-2 gene in N87 (gastric), SK-Br-3 (breast), and SK-OV-3 (ovarian) cell lines and human placenta. DNA was extracted from cell lines and human placenta tissue using guanidine thiocyanate and cesium gradient centrifugation. DNA (15 μg) was cleaved with restriction enzyme HinDIII, separated by electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with radioactive erbB-2 cDNA probe as previously described (26). The cDNA probe corresponds to the entire erbB-2 protein coding region.

Figure 2. Effects of Ab#21 and Ab#23 on the growth of human N87 gastric tumor cells in a monolayer MTT growth assay. A single cell suspension of 10,000 cells/well was plated in a chemically defined medium consisting of RPMI-1640 supplemented with Insulin (5 μg/ml) , human transferrin (10 μg/ml) , 17-ß-estradiol (10 nM), sodium selenite (5 nM), and 10 mM Hepes. PBS, Ab#21, Ab#23 or a combination of Ab#21 and Ab#23 at the indicated concentration were then added. The plates were grown at 37°C in a 5% CO_2 humidified atmosphere. After 7 days, 50 μl

of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.1 mg) were added and allowed to incubate for 4 hours at 37°C. 90% of the media was then removed and the crystals solubilized in 0.175 ml DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. Results are the average of eight wells with standard deviations noted. Under the conditions used, the cell number is directly proportional to MTT reduction.

Figure 3A. Effects of treatment with Ab#21 ( ), Ab#23( ), a combination of Ab#21 and Ab#23 ( ), or PBS ( ) on the growth of N87 tumor xenografts in BNX mice. Tumor cells (5 X 10^6 /mouse) were subcutaneously injected into the flanks of BNX (beige, nude, xid) mice. Treatment begun on day 1 consisted of four trial groups (3 mice per group) each given 0.2 ml intraperitoneal injections twice a week of either PBS ( ) , 200 µg purified Ab#21 (0), 200 µg purified Ab#23 ( ), or a mixture of 100 µg purified Ab#21 and 100 µg of purified Ab#23 ( ) for three weeks. Tumor growth is reported as an average relative tumor volume, s.e.m. ±15%. Two repeats of the experiment gave the same results.

Figure 3B. Effect of treatment after the formation of small tumors. Cells were injected using the same treatment protocol as above except for the fact the treatment was begun 4 days after cell injection instead of 1 day after. Animal care was in accordance with institutional guidelines.

Figure 4A. Effect of antibody binding on erbB-2 protein turnover. Subconfluent N87 cell monolayers were pulse-labeled 1 h with 20 µCi ^{35}S-Cysteine and then chased with 5 mM Cys in the presence of Ab#21 alone, Ab#23 alone, or a 1:1 combination of Ab#21 and Ab#23 (10 µg/ml)for 24 h. Total cellular protein was immunoprecipitated as described in Figure 1 using a monoclonal antibody directed against the

c-terminus of $gp185^{erbB-2}$ coupled to Sepharose and analyzed by SDS-PAGE. The gel was exposed to film at -70°C overnight with an intensifying screen.

Figure 4B. Measurement of tyrosine phosphorylation of $gp185^{erbB-2}$ after incubation with antibody combination. Cells were plated as in Figure 4A. After 1 h cells were processed as in Figure 1B. The proteins were electroblotted onto nitrocellulose paper and incubated with anti-phosphotyrosine IgG (polyclonal, Upstate Biotechnology, Inc.) and immunodetected using an ECL western blotting detection system (Amersham). The film was exposed for 5 min at room temperature.

Figure 5. Effects of Ab#21 and Ab#23 on the growth of human Calu-3 lung adenocarcinoma tumor cells in a monolayer MTT growth assay. A single cell suspension of 10,000 cells/well was plated in a chemically defined medium consisting of RPMI-1640 supplemented with Insulin (5 µg/ml), human transferrin (10 µg/ml) , 17-ß-estradiol (10 nM), sodium selenite (5 nM), and 10 mM Hepes. PBS, Ab#21, Ab#23 or a combination of Ab#21 and Ab#23 at the indicated concentration were then added. The plates were grown at 37°C in a 5% CO_2 humidified atmosphere. After 7 days, 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.1 mg) were added and allowed to incubate for 4 hours at 37°C. 90% of the media was then removed and the crystals solubilized in 0.175 ml DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. Results are the average of eight wells with standard deviations noted. Under the conditions used, the cell number is directly proportional to MTT reduction.

Figure 6. Effects of Ab#23 and Ab#94 on the growth of human Calu-3 lung adenocarcinoma tumor cells in a monolayer MTT growth assay. A single cell suspension of 10,000

cells/well was plated in a chemically defined medium consisting of RPMI-1640 supplemented with Insulin (5 μg/ml), human transferrin (10 μg/ml), 17-β-estradiol (10 nM), sodium selenite (5 nM), and 10 mM Hepes. PBS, Ab#23, Ab#94 or a combination of Ab#21 and Ab#23 at the indicated concentration were then added. The plates were grown at 37°C in a 5% CO_2 humidified atmosphere. After 7 days, 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (0.1 mg) were added and allowed to incubate for 4 hours at 37°C. 90% of the media was then removed and the crystals solubilized in 0.175 ml DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. Results are the average of eight wells with standard deviations noted. Under the conditions used, the cell number is directly proportional to MTT reduction.

Figure 7. The cDNA sequence for the *single chain anti-erbB2 antibody, Ab#23.

Figure 8. The cDNA sequence for the *single chain anti-erbB2 antibody, Ab#21 (e22).

DETAILED DESCRIPTION OF THE INVENTION

One object of the present invention is a combination of at least two monoclonal antibodies, which is capable of preventing and treating human malignancies, wherein the malignant cells overexpress $gp185^{erbB-2}$ and wherein said at least two different antibodies each recognize a different epitope of the gp185 expression product of erbB-2, therefor the antibodies do not cross react with each other. An embodiment of the present invention provides for the combination to comprise first and second antibodies which are preferably combined such that the resulting ratio of the first to second is effective for decreasing the expression product of the erbB-2 gene. A convenient method for

measuring the expression product of erbB-2 gene may be found in Figure 4A. The decrease in the expression of the erbB-2 gene product is the result of the combination decreasing the half life of erbB-2 protein in the cell. In another embodiment of the present invention, the combination of the antibodies has the characteristic trait of essentially not increasing the tyrosine phosphorylation of gp185 expression product.

An example of a first to second antibodies ratio having the activity necessary to decrease the expression product of the erbB-2 gene comprises a ratio of from about 1:2 to about 2:1. Preferably, such a ratio is 1:1. The present invention, however, is not intended to be limited to the antibody ratios discussed herein. The fact that other ratios are effective and may yield higher activity than the 1:1 ratio used as an example is recognized and acknowledged by the inventors as being within the scope of this invention.

The activity of this combination is exemplified in Figures 1A-C, 2 and 3A, B as follows. Figure 1A-C demonstrate that the N87 cells overexpress the gp185 erbB-2 protein as a result of erbB-2 gene amplification. Figure 2 shows that a combination of Ab#21 and Ab#23 inhibits the growth of N87 cells <u>in vitro</u>. Similar results have been demonstrated using the combination of Ab#23 and Ab#94 as well as Ab#23 and Ab#21, on the growth of human Calu-3 lung adenocarcinoma (See Figures 5 and 6). Figure 3A and B show the activity of combinations of Ab#21 and Ab#23 inhibiting and reversing the growth of N87 cells growing as tumors in immunodeficient mice. These results indicate the general nature of the application of combinations of antibodies.

The antibodies against the erbB2 gene encoded product used in this invention can be designed as chimeric

antibodies. Chimeric antibodies have variable regions (antigen binding regions) of nonhuman (e.g., murine) origin and constant regions of human origin. Because they are predominantly human, chimeric antibodies are less immunogenic in humans, which can help overcome problems associated with administering foreign proteins to humans.

In addition, the antibodies of the present invention may be produced through genetic recombination or the Kohler-Milstein hybridoma method for production of antibodies. It is also recognized that fragments, analogues or derivatives of the antibodies themselves can be utilized in this invention in place of the entire antibody.

Another object of the present invention provides for antibodies against erbB-2 gene encoded product which are designed as single chain antibodies. A single chain antibody is one in which the light and heavy variable regions of the antibody are linked together to form a single chain antibody. It is contemplated in this application that a combination of these antibodies include antibodies which are combined as an admixture as discussed above and antibodies which are combined to form a bispecific antibody.

A bispecific antibody is an artificially produced antibody usually comprised of two single chain antibodies each of which is recognizes a different antigen binding site.

The following are examples of some of the human malignancies which may be treated or prevented using the present invention; adenocarcinonas of the breast, ovary, lung and stomach.

Another embodiment of applicants' invention provides a method for preventing and eradicating the human malignancies described above. The method involves administering to a patient an effective dose of a combination of anti-erbB-2

antibodies to achieve an effective concentration of the antibody combination at the tumor site; for example, a concentration of at least 1μg/ml. Preferably the concentration at the tumor site does not exceed about 10μg/ml. In general, in order to achieve the desired concentration of the combination at the tumor site the combination is administered in a dose from about .1 mg/kg to about 10 mg/kg of body weight.

Another embodiment of Applicants' invention provides for the antibody combination to be used in passive tumor therapy, wherein an effective dose of the antibody combination is administered in or with a pharmaceutically acceptable vehicle to a patient afflicted with a human malignancy overexpressing gp185^{erbB-2}. Examples of vehicles are non-toxic buffers, physiological saline, etc.

Applicants' invention also provides for at least one of the antibodies of the antibody combination to be used as a component of an immunotoxin. For immunotoxin therapy, at least one antibody of the combination can be linked to an anti-cancer pharmaceutical or a cytotoxin to form an immunotoxin. Various pharmaceutical or cytotoxic agents can be chemically or genetically coupled to the combination. Examples of some useful therapeutic agents include: radioactive compounds (e.g., isotopes of Boron and Rhenium); agents which bind DNA, such as alkylating agents or various antibodies (e.g., daunomysin, adriamycin, chlorambucil); anti-metabolites (e.g., methotrexate); and inhibitors of protein synthesis (e.g., diphtheria toxin and toxic plant proteins). The use of the combination, wherein at least one of the antibodies in the combination is coupled to an immunotoxin will increase the efficacy of the therapy.

Administration to a patient of an effective dose of the combination of antibodies described herein may be

accomplished via chronic intraveneous administration for a period of time sufficient to result in the regression or eradication of the human malignancy being treated. Administration of the combination may also be accomplished in a patient by direct injection or delivery of the combination to the tumor site. Such administration would be of sufficient duration and concentration to result in eradication or reduction of the tumor.

Although the scope of the present invention is not intended to be limited to any theoretical reasoning, the following theories may explain a mechanism by which down regulation and protection from human malignant cells overexpressing $gp185^{erbB-2}$ is achieved by the two antibody combination.

One mechanism which has been postulated is that the two antibody combination acts by constraining $gp185^{erbB-2}$ into an activated conformation thus mimicking an agonist ligand. If the two antibody combination mimics the ligand, then treatment using the combination should result in increased $gp185^{erbB-2}$ autophosphorylation. Anti-phosphotyrosine immunoblots were used to test this hypothesis. As shown in Figure 4B, no increase in tyrosine phosphorylation of $gp185^{erbB-2}$ from N87 cells was observed 1 or 2 hours after the addition of the antibody combination or up to 24 h of treatment. This suggests that the antibody combination does not increase the autophosphorylation of $gp185^{erbB-2}$ and therefore does not act to inhibit the activity of the tyrosine kinase.

The results demonstrate that a combination of anti-receptor antibodies leads to different and more potent anti-tumor activities than single antibodies. More specifically, results indicate that the combination antibody therapy is a useful approach to treatment of human

malignancies overexpressing gp185^{erbB}. This approach may be particularly important in the treatment of gastric cancer, a disease which responds poorly to current systemic chemotherapies.

The present invention is further illustrated by the following Examples which are not intended to limit the scope of the invention.

EXAMPLE 1

Preparation of Antibodies

As a source of human erbB-2 protein we used a NIH/3T3 cell engineered to express the human erbB-2 protein on its surface (N/erbB-2). Membrane preparations of these cells were prepared by hypotonic lysis in 2mM Hepes pH 7.4, removal of nuclei by centrifugation at 5,000 x g and isolation of membranes by centrifugation at 100,000 x g. Mice were immunized with 100µg of N/erbB-2 membran preparation in a 50:50 mix of adjuvant in 200µl. Adjuvant was Freund's complete for the first injection followed by Freund's incomplete adjuvant. Mice were given 4 intraperitoneal injections over 4 weeks. One week following the last boost sera was obtained and an anti-erbB-2 immune response was determined to exist by immunoprecipition analysis of gp185 erbB-2 protein from metabolically labeled cells. An immune mouse was then selected and boosted with 100µg of N/erbB-2 membrane preparation and fusion with Ag8.653 myelomea cells conducted according to standard methods. Selection of hybrid clones was by resistence to hypoxanthine, aminopterin, and thymine (HAT) containing media again according to standard methods. Identification of hybridomas secreting an anti-erbB-2 monoclonal antibody was by ELISA using as antigen N/erbB-2 membrane protein attached to 96 well dishes. ELISA reaction was developed

using peroxidase coupled goat anti-mouse antibody and standard methods. Hybridoma cultures secreting an anti-erbB-2 antibody were subjected to two rounds of singl cell cloning and identification of positive subclones by ELISA as described above.

The above procedure is used to produce three monoclonal antibodies designated as Ab#21; Ab#23; and Ab#94.

Monoclonal antibodies directed against the extracellular domain of $gp185^{erbB-2}$ were tested for specific reaction to N/erbB-2 cell membranes in an ELISA assay. Two of these designated Ab#21 and Ab#23 after screening in growth assays exhibited the highest biological activity and were used in this study. Antibodies were isolated in large amounts from ascites fluid and purified by HPLC with a Gammabind Ultra column (Genex, Gaithersburg, MD). Standard SDS-PAGE gel electrophoresis was run under non-reducing conditions using Coomassie blue staining with a single band of 130 kd observed indicating a >98% purified preparation (data not shown).

Both antibodies specifically immunoprecipitated a single ^{35}S-labeled protein of MW 185,000 from SK-Br-3 cells (a breast cancer cell line which overexpresses $gp185^{erB-2}$ protein) (22) as shown in Figure 1A. No immunoprecipitation was detected in cells which do not overexpress the $gp185^{erbB-2}$ protein (e.g. MDA-MB-468, data not shown).

The effect of these antibodies on cell proliferation was studied on a gastric cell line, N87, which overexpresses $gp185^{erbB-2}$ at levels commensurate with SK-Br-3. An immunoblot of the N87 cell line and a nude mouse tumor xenograft from N87 is shown in Figure 1B compared to the breast cell lines SK-Br-3 (high level of $gp185^{erbB-2}$ overexpression) and MDA-MB-231 (low level of $gp185^{erbB-2}$ overexpression) The levels of erbB-2 gene amplification in

N87 as shown in Figure 1C surpassed those found in the well characterized SK-Br-3 and SK-OV-3 cell lines (22).

EXAMPLE 2

Effect of Antibodies on Tumor Growth In Vitro

The effect of these antibodies on growth was first studied *in vitro* using a semiautomated colorimetric MTT assay. A single cell suspension of 10,000 cells/well was plated in a chemically defined media consisting of RPMI-1640 supplemented with insulin, human transferrin, 17 -estradiol, sodium selemite and Hepes buffer. PBS, Ab#21, Ab#23 or a combination of Ab#21 and Ab#23 were then added. The cells were allowed to grow at 37°C in a 5% CO_2 humidified atomosphere. After 7 days, 3- (4,5-dimethylthiazol-2-y)-2,5-diphenyl tetrazolium bromide (MTT reagent) was added and allowed to incubate for 4 hours at 37°C, 90% of the media was then removed and the crystals solubilized in DMSO. Optical densities were measured at 540 nm in a molecular devices Vmax kinetic microplate reader. A dose response analysis of the effects of the antibodies on N87 cell proliferation is shown in Figure 2. Antibodies Ab#21 or Ab#23 administered individually had no effect on the monolayer growth of cells up to a concentration of 10 µg/ml (6 µM). Administration of a 1:1 combination of Ab#21 and Ab#23, however, markedly affected cell proliferation at doses as low as 1 µg/ml. Fab fragments prepared from both antibodies also had no effect on cell growth alone or in combination (data not shown). In analogous experiments with three other gastric cell lines displaying little or no overexpression by immunoblot analysis, no inhibition of growth even at the highest dose was observed with the antibody combination or the antibodies alone.

EXAMPLE 3

Preventive Combination Antibody Therapy

The efficacy of combination antibody therapy was tested on the growth of N87 tumor xenografts. One inoculation of five million N87 cells were injected subcutaneously into nude mice produce rapidly growing tumors with a short latency. Tumor growth at the injection site was easily quantitated. As shown in Figure 3A, the N87 cells did not form tumors in the animals treated twice a week for three weeks with a total of 200 µg of antibodies per injection with the combination of Ab#21 and Ab#23. In sharp contrast they were potently tumorigenic in animals treated with the single antibodies or PBS and the tumor grew to over 1 cm^3 in tumor volume over the period measured. In contrast to *in vitro* experiments, each monoclonal antibody alone may have limited activity to partially restrict the rate of tumor growth. However, the activity exhibited by the combination far exceeded the cumulative effect expected from the combination.

To determine if the combined therapy with Ab#21 and Ab#23 was able to eradicate established tumors, an experiment was performed in which tumors were allowed to grow to measurable sizes prior to antibody treatment. The results are illustrated in Figure 3B. In animal groups randomized so that the starting size of the tumors was near the same volume (100 mm^3), the tumors continued to grow when the animals were given single antibody treatment of Ab#21 or Ab#23 (200 µg/injection, 2 injection/week, 3 weeks, 6 mice). In contrast, in the animals given two antibody combination treatment of Ab#21 and Ab#23, results shown are the average of 6 animals, tumors completely regressed after 11 days (4 treatments of 200 µg of total antibody) . This is the first reported observation of tumor xenograft regression induced

by a combination of anti-erbB-2 monoclonal antibodies. Previous studies have shown that two anti-neu antibodies can inhibit the growth of tumors by murine cells transformed by the mutationally activated neu oncogene (23). The activation of the murine neu oncogene is accomplished by point mutation as evidenced by qualitative interference in the structure and function of the neu gene, whereas the human erbB-2 oncogene is activated by overexpression of erbB-2, a quantitative interference of the apparently normal protein which results in tumor formation.

Since the mechanisms for tumor growth are so different between murine and human, it is totally unexpected that similar mechanisms of neutralization of the genes involved would be effective. This effect is also seen with the inhibition of leukemic tumor cell growth using anti-transferrin monoclonal antibodies (24).

EXAMPLE 4

Antiproliferative Effects of Antibody Combination

To investigate the molecular basis for the antiproliferative effects of Ab#21 and Ab#23, we measured the rate of $gp185^{erbB-2}$ turnover in the presence or absence of antibodies. N87 cells were pulse-labeled with ^{35}S-Cys and then chased for various times in the presence of single antibody or the Ab#21/#23 combination. The results of a 24 h chase are shown in Figure 4A. The antibody $gp185^{erbB-2}$ combination induced rapid degradation of $gp185^{erbB-2}$ while the individual antibody treatment had little or no effect. Thus, the antiproliferative effect of Ab#21/Ab#23 treatment might likely be explained by their ability to increase the turnover of $gp185^{erbB-2}$.

EXAMPLE 5

Combination Antibody Treatment Effects the Growth of Calu-3 Cells

In order to demonstrate that the effect of combination of anti-erbB-2 antibodies Ab #21 and Ab #23 is not limited to effect on the N87 gastric cancer cell line, we investigated the human lung adenocarcinoma cell line Calu-3. This cell overexpresses the gp185 erbB-2 protein as determined by immunoblot analysis (data not shown). In experiments very similar to that described above, the combination of Ab #21 and Ab #23 show dramatic inhibition of cell growth as measured in an MTT assay (figure 5). In this experiment, a single cell suspension of 10,000 cells/well was plated in a chemically defined media consisting of RPMI-1640 supplemented with insulin, human transferrin, 17-estradiol. sodium selenite, and Hepes buffer. PBS, Ab#21, Ab#23 or a combination of Ab#21 and Ab#23 were then added. The cells were allowed to grow at 37°C in a 5% CO_2 humidified atmosphere. After 7 days, MTT reagent was added and allowed to incubate for 4 hours at 37°C. 90% of the media was then removed and the crystals solubilized in DMSO. Optical densities were measured at 540 nm in a Molecular Devices Vmax kinetic microplate reader. A dose response analysis of the effects of antibody treatment is shown in figure 5. This result indicates that combination antibody therapy is not limited in effectiveness to N87 cells or gastric cancer cells. It also indicates that combination antibody therapy may have effectiveness in the treatment of adenocarcinoma of the lung.

EXAMPLE 6

Effectiveness of Other Combinations of Antibodies in Inhibiting Cell Growth

In order to determine if Ab#21 and Ab#23 are unique in their ability to combine to cause growth inhibition, we investigated the combination of Ab#94 and Ab#23 on the growth of Calu-3 cells in vitro. An MTT assay of cell growth was conducted as described in EXAMPLE 5. As shown in figure 6, the combination of antibodies inhibits cell growth and the individual antibodies do not. This indicates that the ability to combine antibodies to produce a more profound growth inhibition is not limited to a particular antibody combination.

Antibodies #21; Ab#23; and Ab#94 have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, USA. Ab#21 was deposited on ________________ and given ATCC #________. Ab#23 was deposited on ________________ and given ATCC #________. Ab#94 was deposited on ____________ and given ATCC #________.

While the present invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art in view of the foregoing description. Accordingly, the invention is intended to embrace all such alternatives, modifications and variations in following within the broadest scope and spirit of the following claims.

Example 7

Generation of a single chain (Fv) from mAb e23

Poly A RNA was extracted from hybridoma cells using oligo dT affinity chromatography (In vitrogen). cDNA was prepared using random primer (N_6) (Boerhinger Mannheim). The immunoglobulin light and heavy chain clones were isolated using PCR and the primers: light chain, 5' CAC GTC GAC ATT CAG CTG ACC CAC TCT CCA and GAT GGA TCC AGT TGG TGC AGC ATC3'; heavy chain 5'C GGA ATT TCA GGT TCT GCA GIA GTC

WGG3' and 5' AGC GGA TCC AGG GGC CAG TGG ATA GAC3' [G,A,C,T stand for standard nucleotides; I for inosine, W for A or T]. The products of the PCR reaction wre cloned into PUC18. Linkage into a SC(Fv) was by PCR giving the individual light and heavy cDNA clones and 4 oligonucleotides

5' - cgagatgagtccagctgacccagtctc

5' - gaagatttaccagaaccagaggtagaaccttttatttccagcttgga

5' - ctggttctggtaaatcttctgaaggtaaaggtgtgcagctgcaggag

5' - cgagtgcaagcttaggagacggtgaccgt .

The light and heavy chain coding regions were joined by a synthetic linker GSTSGSGKSSEGKG specified by overlapping oligonucleotides as described. The intact scFv coding region was inserted in frame with an E.coli OMPA leader sequence under direction of the lambda P_L promoter. Induction of protein and bacterial lysis and refolding was as previuosly described (28). scFv was purified as a single peak from CM chromatography and judged to be >70% by SDS gel electrophoresis.

Example 8

Generation of a scFv from mAb e21

Poly A RNA was extracted from hybridoma cells using oligo dT affinity chromatography (In vitrogen). cDNA was prepared using random primer (N_6) (Boerhinger Mannheim). The immunoglobulin light and heavy chain clones were isolated using PCR and the primers: light chain, 5' CAC GTC GAC ATT CAG CTG ACC CAC TCT CCA and GAT GGA TCC AGT TGG TGC AGC ATC3'; heavy chain 5'C GGA ATT TCA GGT TCT GCA GIA GTC WGG3' and 5' AGC GGA TCC AGG GGC CAG TGG ATA GAC3' [G,A,C,T stand for standard nucleotides; I for inosine, W for A or T]. The products of the PCR reaction wre cloned into PUC18. Linkage into a scFv was by PCR giving the individual light and heavy cDNA clones and 4 oligonucleotides

5' - cgagatgagtccagctgacccagtctc

5' - gaagatttaccagaaccagaggtagaaccttttatttccagcttgga
5' - ctggttctggtaaatcttctgaaggtaaaggtgtgcagctgcaggag
5' - cgagtgcaagcttaggagacggtgaccgt.

The light and heavy chain coding regions were joined by a synthetic linker GSTSGSGKSSEGKG specified by overlapping oligonucleotides as described. The intact scFv coding region was inserted in frame with an E.coli OMPA leader sequence under direction of the lambda P_L promoter. Induction of protein and bacterial lysis and refolding was as previuosly described (28). scFv was purified as a single peak from CM chromatography and judged to be >70% by SDS gel electrophoresis.

REFERENCES

1. C.R. King, M.H. Kraus, S.A. Aaronson, Science 229, 974 (1985).
2. D.J. Slamon, et al., Science 235, 177 (1987).
3. D.J. Slamon, et al., Science 244, 707 (1989).
4. J. Yokota, et al., Lancet 1, 765 (1986).
5. C.R. King, et al., Cancer Res. 49, 4185 (1989).
6. P.M. Schneider, et al., Cancer Res. 49, 4968 (1989).
7. J. Park, et al., Cancer Res. 49, 6605 (1989).
8. C. Wright, et al., Cancex- Res. 49: 2087 (1989).
9. A.K. Tandon, et al., J. Clin. Oncol. 7, 1120 (1989).
10. S. Paik, et al., J. Clin. Oncol. 8, 103 (1990).
11. A. Borg, et al., Cancer Res. 50, 4332 (1990).
12. A. Berchuck, et al., Cancer Res. 50, 4087 (1990).
13. Y. Yonemura, et al., Cancer Res. 51, 1034 (1991).
14. J.A. Kern, et al., Cancer Res. 50, 5184 (1990).
15. P.P. Di Fiore, et al., Science 237, 178 (1986).
16. R.M. Hudziak, J. Schlessinger, A. Ullrich, Pro. Natl. Acad. Sci. U.S.A. 84, 7159 (1987).
17. Pierce, et al., oncogene, in press.
18. Y. Yarden, A. Ullrich, Biochem. 27, 3114 (19.88).
19. J. Schlessinger, Biochem. 27, 3119 (1988).
20. R.M. Hudziak, et al., Mol. Cell. Biol. 9, 1165 (1989).
21. C.R. Divgi, et al., JNCI 83, 97 (1991).
22. M. H. Kraus, N. C. Popescu, S. C. Amsbaugh, C. R. King, EMBO J. 6, 605 (1987).
23. J.A. Drebin, V.C. Link, M.I. Greene, Oncogene 2, 273 (1988).
24. S. White, et al., Cancer Res. 50, 6295 (1990).
25. R. Kumar, H.M. Shepard, J. Mendelsohn, Mol. Cell. Biol. 11, 979 (1991).

26. C.R. King, et al., Cancer Res. 49,4185 (1989).
27. M.C. Hancock, et al., Cancer Res. 51,4575-4580 (1991).
28. M.W. Pantaliano, et al., Biochemistry 30, 117-125 (1991).

WE CLAIM:

1. An antibody combination for treating or preventing human malignancies wherein the malignant cells overexpress erbB-2, said combination comprising:

at least two different monoclonal antibodies, each of which recognizes a different epitope of the gp185 expression product of erbB-2.

2. An antibody combination as in Claim 1, wherein said combination comprises:

first and second different monoclonal antibodies, and wherein said combination decreases the expression product of the erbB-2 gene.

3. An antibody combination as in Claim 2, wherein said combination does not essentially increase tyrosine phosphorylation of the gp185 expression product.

4. An antibody combination of Claim 2, wherein at least one of said antibodies in said antibody combination has been linked to an immunotoxin molecule.

5. A method of treating a patient afflicted with a human malignancy wherein the malignant cells overexpress erbB-2, the method comprising:

administering to the patient afflicted with said human malignancy an effective amount of a combination of at least two different monoclonal antibodies which recognize different epitopes of the gp185 expression product of erbB-2.

6. A method as in Claim 5, wherein said combination comprises:

first and second different monoclonal antibodies and wherein said combination decreases the expression product of the erbB-2 gene.

7. A method as in Claim 6, wherein said combination does not essentially increase tyrosine phosphorylation of the gp185 expression product.

8. A method as in Claim 6, wherein said effective amount provides a concentration of at least 1 μg/ml at the tumor site.

9. A method as in Claim 8, wherein said effective amount provides a concentration of not more than 10 μg/ml at the tumor site.

10. A method as in Claim 6, wherein said effective amount is a dose of from about .1 mg/kg to about 10 mg/kg of body weight of the patient.

11. A method as in Claim 6, wherein at least one of said antibodies of said combination has been linked to an immunotoxin molecule.

12. A composition capable of preventing or treating human malignancies wherein the malignant cells overexpress erbB-2 comprising:

(a) an effective amount of a combination of at least two different monoclonal antibodies which recognize different epitopes of the gp185 expression product of erbB2; and

(b) a pharmaceutically acceptable carrier.

FIGURE 1A

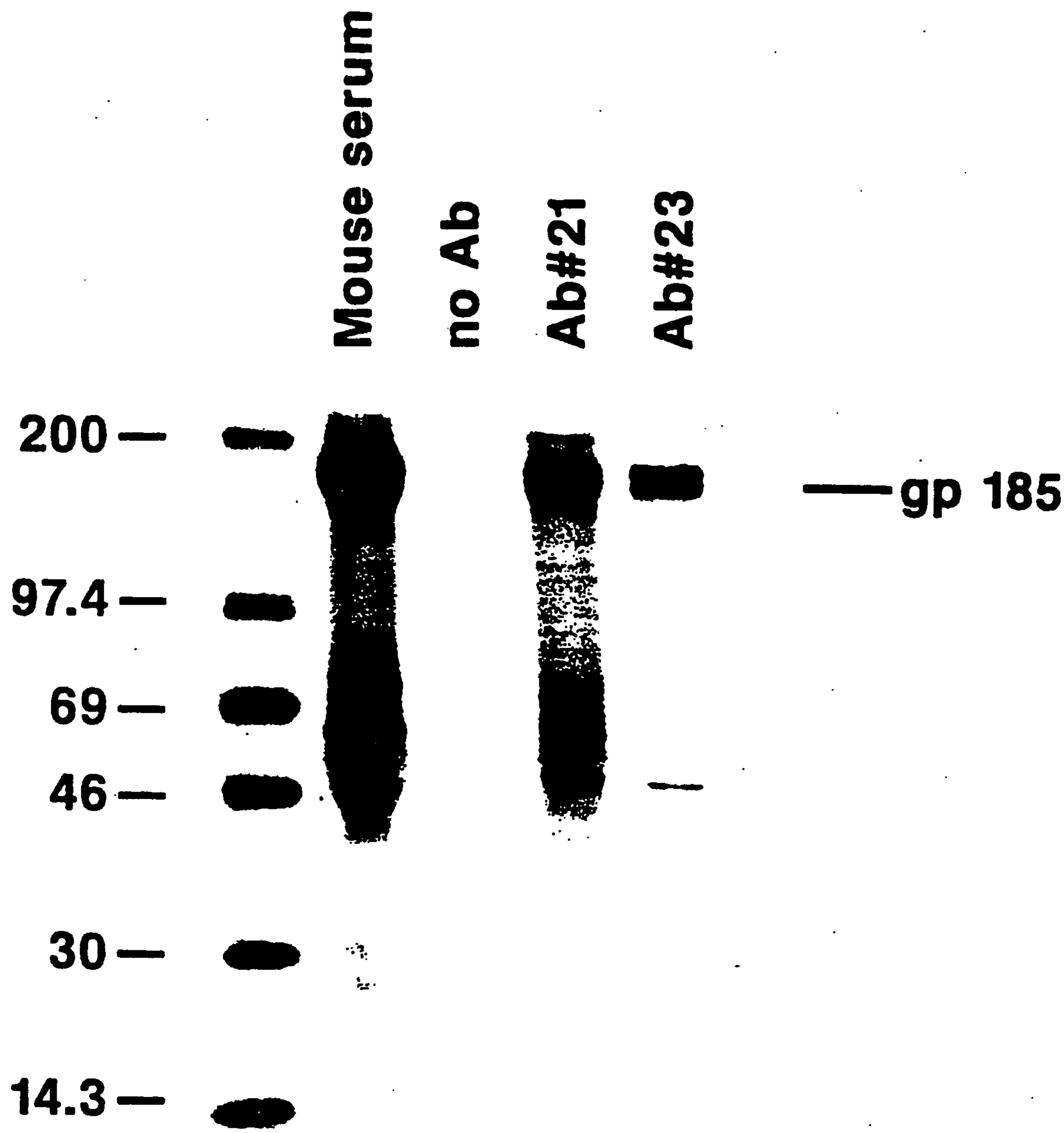


FIGURE 1B

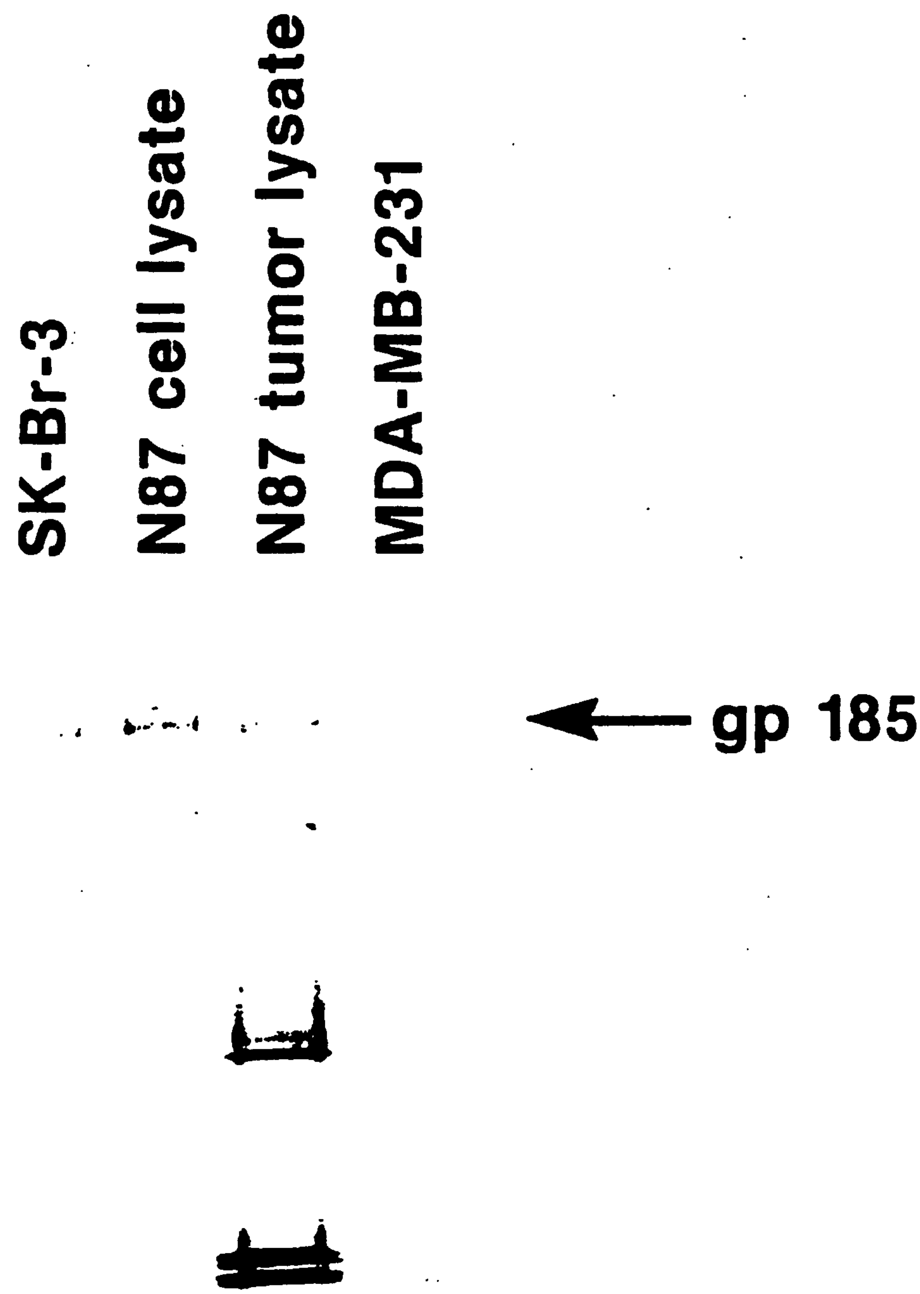


FIGURE 1C

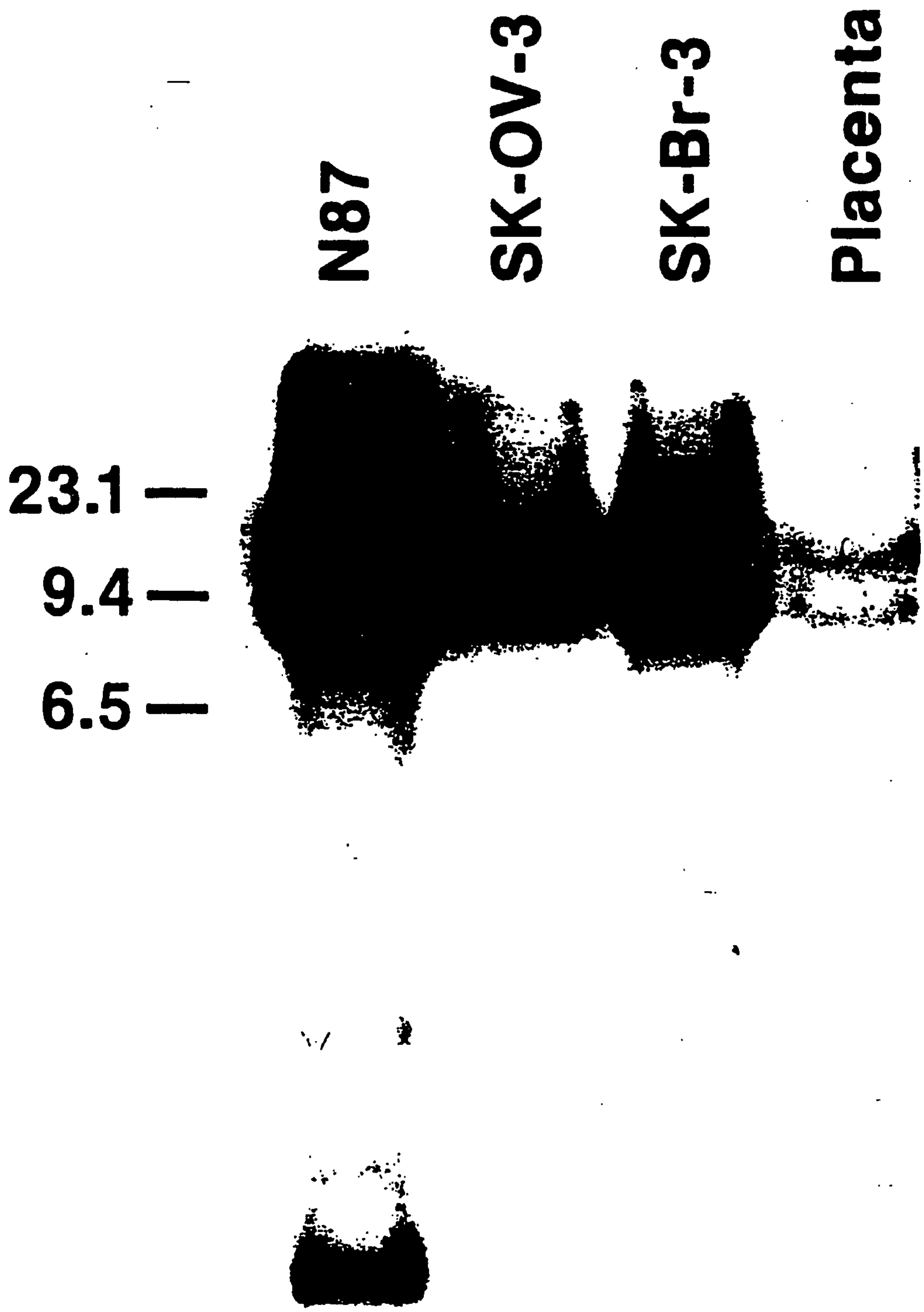
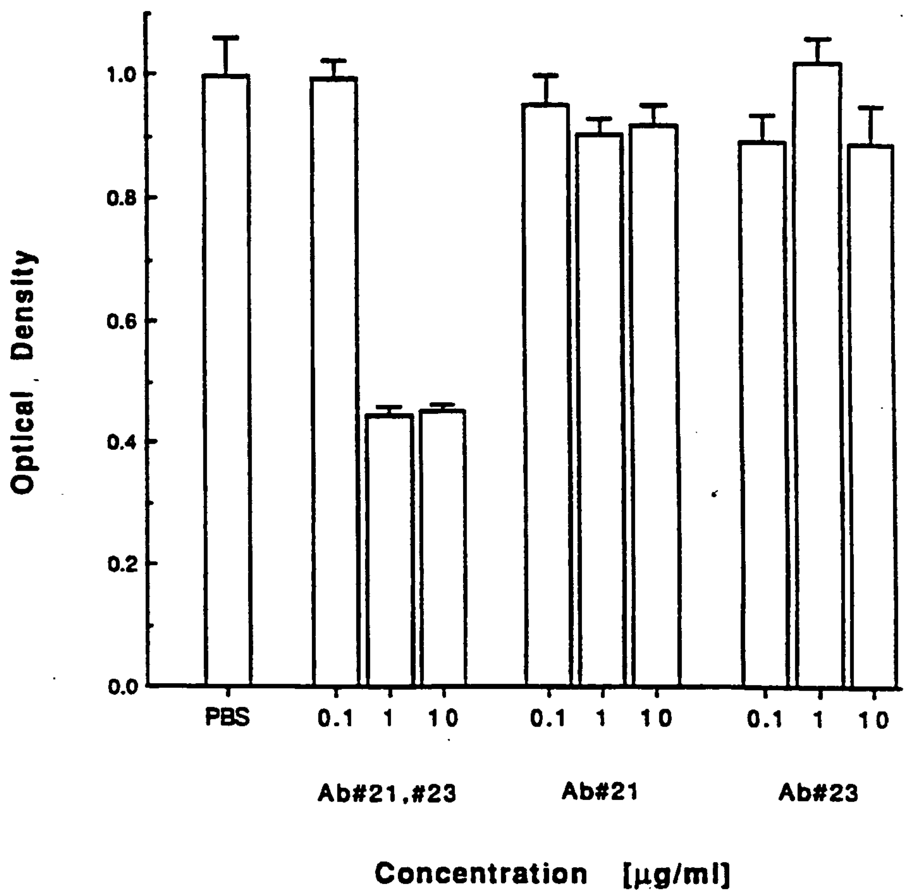


FIGURE 2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0.4
0.2
0.0
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0.1 1 10
0.1 1 10
Ab#21,#23
Ab#21
Ab#23
Concentration [µg/ml]

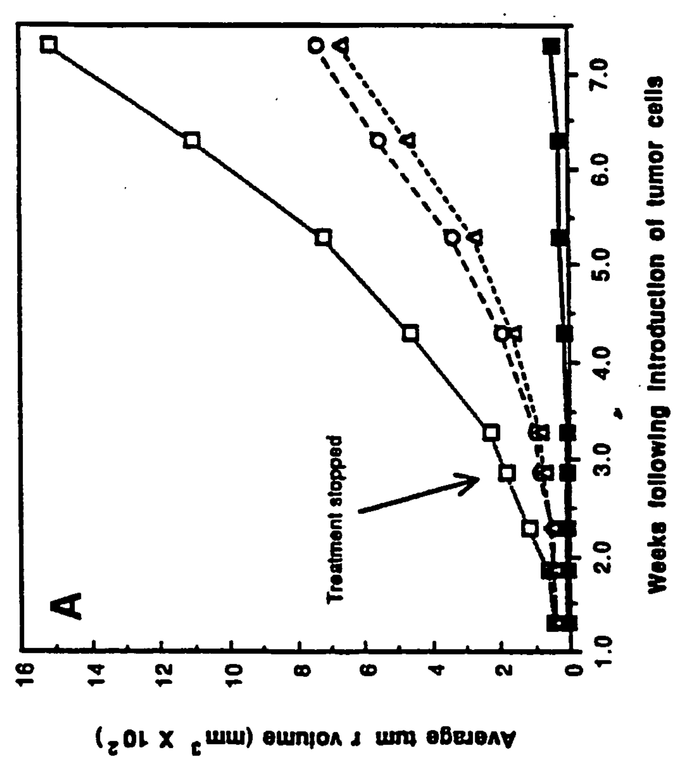


FIGURE 3A

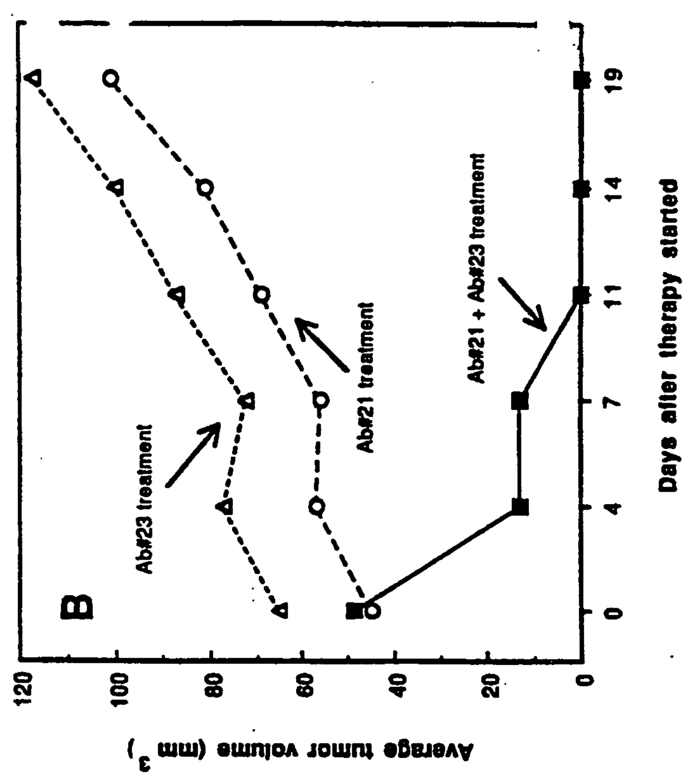


FIGURE 3B

FIGURE 4A

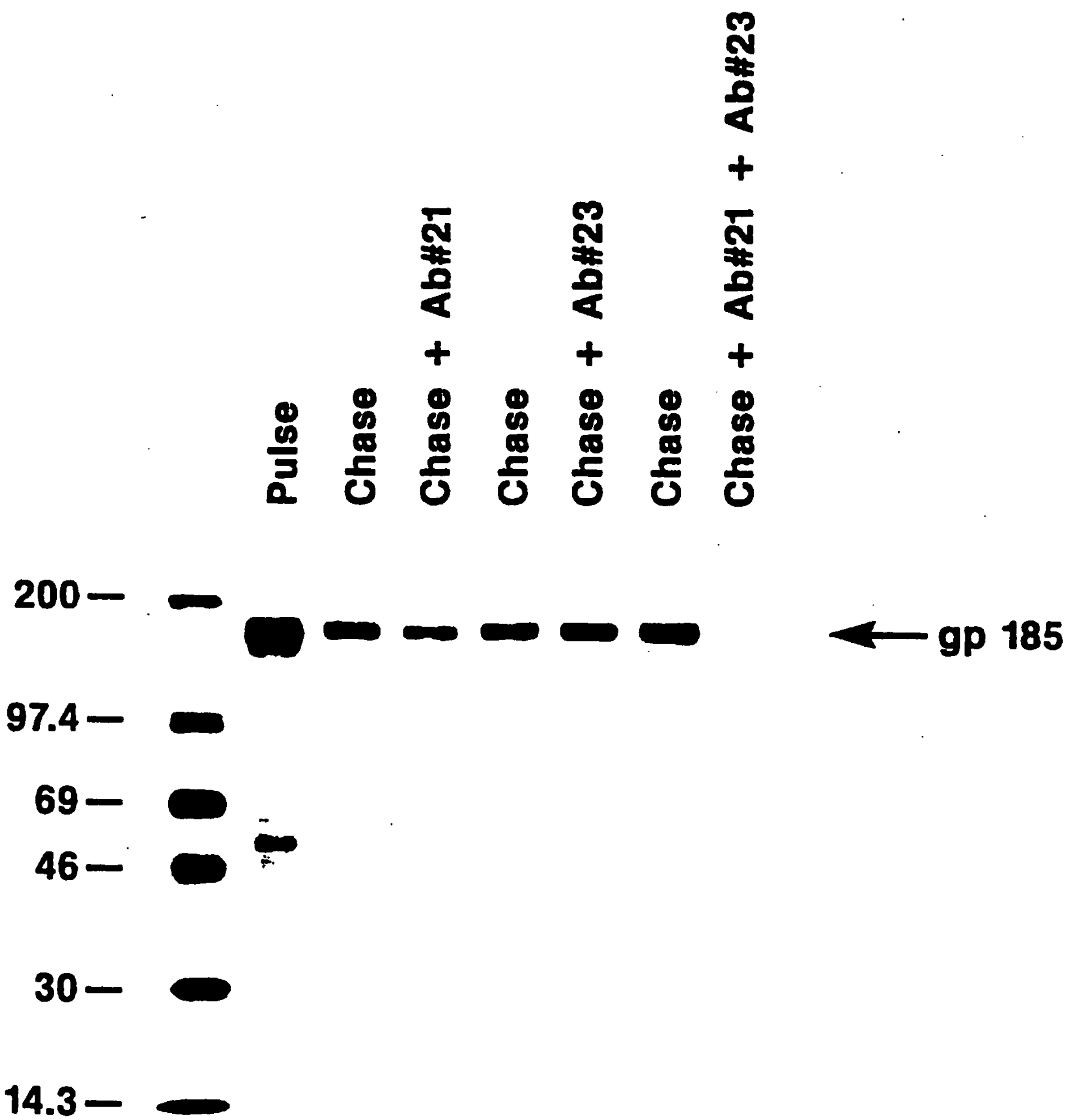

Pulse
Chase
Chase + Ab#21
Chase
Chase + Ab#23
Chase
Chase + Ab#21 + Ab#23
200
97.4
69
46
30
14.3
gp 185

FIGURE 4B

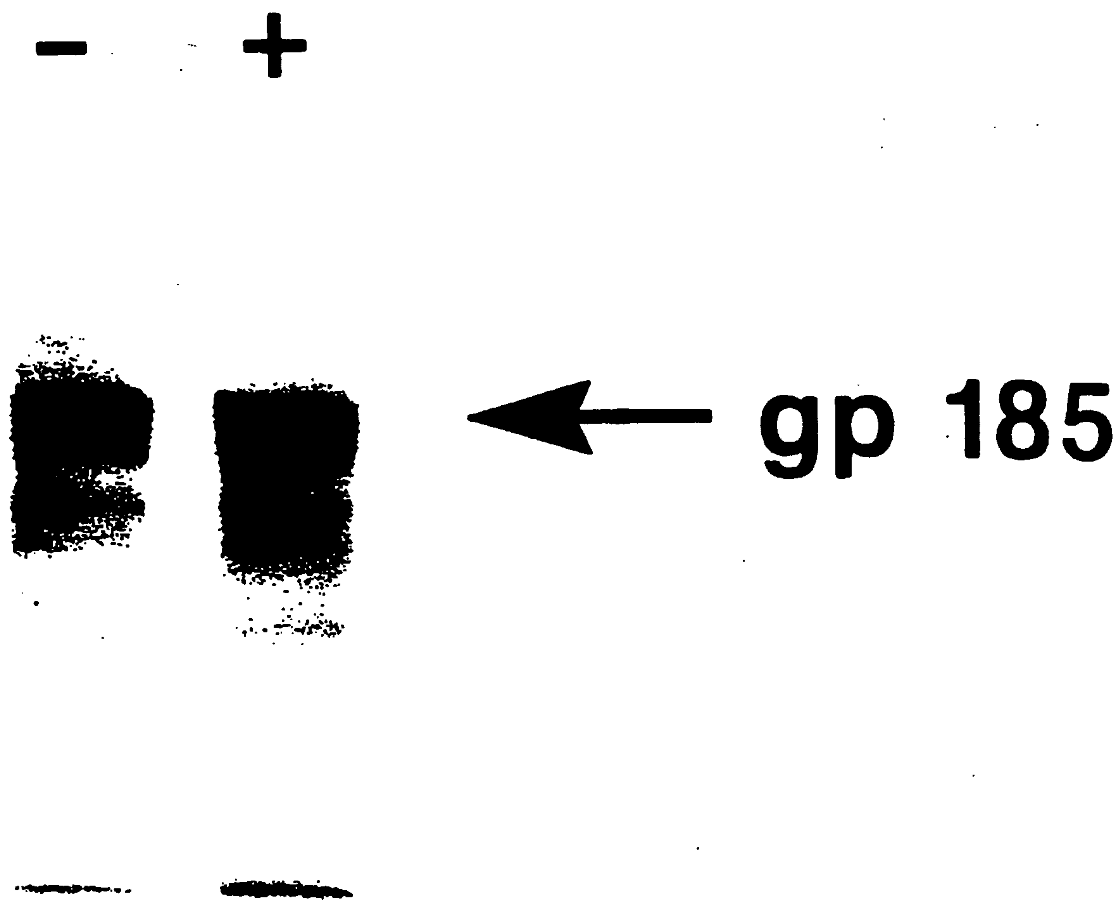
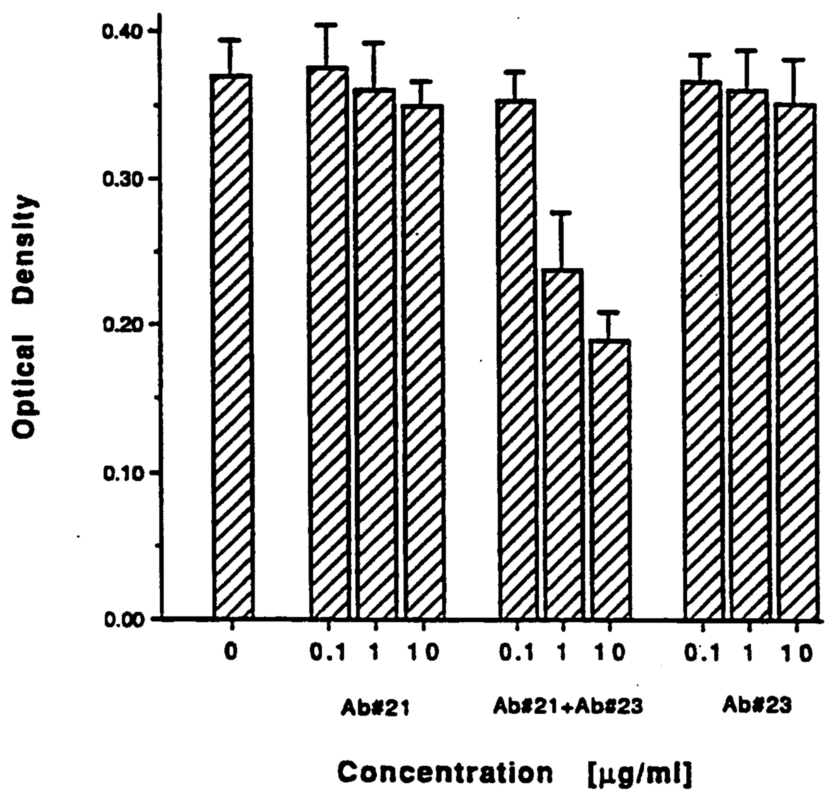


FIGURE 5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0.30
0.20
0.10
0.00
Optical Density
0
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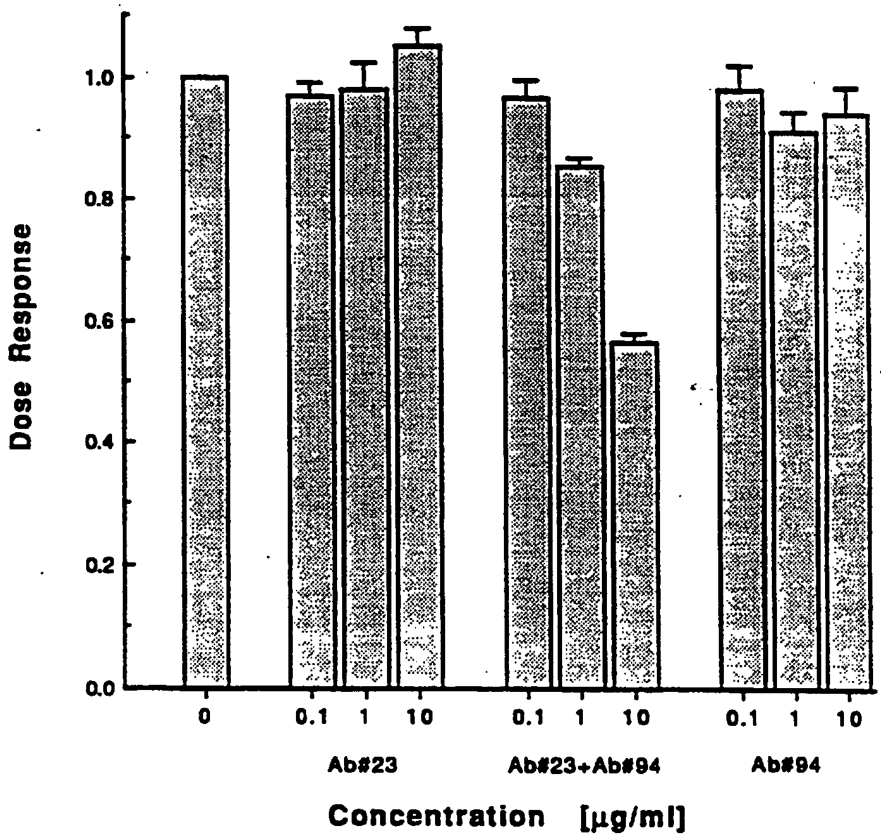
0.1 1 10
0.1 1 10
Ab#21
Ab#21+Ab#23
Ab#23
Concentration [μg/ml]

FIGURE 6


1.0
0.8
0.6
0.4
0.2
0.0
Dose Response
0
0.1
1
10
0.1
1
10
0.1
1
10
Ab#23
Ab#23+Ab#94
Ab#94
Concentration [μg/ml]

ATGGACCTGCAGCTGACCCAGTCTCCAGCAATCCTGTCTGCATCTCCAGG
MetAspLeuGlnLeuThrGlnSerProAlaIleLeuSerAlaSerProGly

GGAGAAGGTCACAATGACTTGCAGGGCCACCCCAAGTGTAAGTTACATGC
GluLysValThrMetThrCysArgAlaThrProSerValSerTyrMetHis

ACTGGTATCAGCAGAAGCCAGGATCCTCCCCCAAACCTTGGATTTATACC
TrpTyrGlnGlnLysProGlySerSerProLysProTrpIleTyrThr

ACATCCAACCTKGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCGGTGGGTC
ThrSerAsnLeuAlaSerGlyValProAlaArgPheSerGlyGlyGlySer

TGGGACCTCTTACTCTCTCACAGTCAGCAGAGTGGAGGCTGAAGATGCTG
GlyThrSerTyrSerLeuThrValSerArgValGluAlaGluAspAlaAla

CCACTTATTACTGCCAGCAGTGGAGTCGTAGCCCACCCACGTTCGGAGGG
ThrTyrTyrCysGlnGlnTrpSerArgSerProProThrPheGlyGly

GGGTCCAAGCTGGAAATAAAAGGTTCTACCTCTGGTTCTGGTAAATCTTC
GlySerLysLeuGluIleLysGlySerThrSerGlySerGlyLysSerSer

TGAAGGTAAAGGTGTGCAGCTGCAGGAGTCAGGACCTGAGGTGGTGAAGC
GluGlyLysGlyValGlnLeuGlnGluSerGlyProGluValValLysPro

CTGGAGGTTCAATGAAGATATCCTGCAAGACTTCTGGTTACTCATTCACT
GlyGlySerMetLysIleSerCysLysThrSerGlyTyrSerPheThr

GGCCACACCATGAACTGGGTGAAGCAGAGCCATGGAAAGAACCTTGAGTG
GlyHisThrMetAsnTrpValLysGlnSerHisGlyLysAsnLeuGluTrp

GATTGGACTTATTAATCCTTACAATGGTGATACTAACTACAACCAGAAGT
IleGlyLeuIleAsnProTyrAsnGlyAspThrAsnTyrAsnGlnLysPhe

TCAAGGGCAAGGCCACATTTACTGTAGACAAGTCGTCCAGCACAGCCTAC
LysGlyLysAlaThrPheThrValAspLysSerSerSerThrAlaTyr

ATGGAGCTCCTCAGTCTGACATCTGAGGACTCTGCAGTCTATTACTGTGC
MetGluLeuLeuSerLeuThrSerGluAspSerAlaValTyrTyrCysAla

AAGGAGGGTTACGGACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGG
ArgArgValThrAspTrpTyrPheAspValTrpGlyAlaGlyThrThrVal

TCACCGTCTCC
ThrValSer

FIGURE 7

Figure 8

ATGCAGCTGACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAAAA 50
MetGlnLeuThrGlnSerProAlaIleMetSerAlaSerProGlyGluLys

GGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTAACATGCACTGGT 100
ValThrMetThrCysSerAlaSerSerSerValSerAsnMetHisTrpTyr

ATCAGCAGAAGTCAAGCACCTCCCCCAAACTCTGGGTTTATGACACATCC 150
GlnGlnLysSerSerThrSerProLysLeuTrpValTyrAspThrSer

AAACTGGCTTCTGGAGTCCCAGGTCGCTTCAGTGGCAGTGGGTCTGGAAA 200
LysLeuAlaSerGlyValProGlyArgPheSerGlySerGlySerGlyAsn

CTCTTACTCTCTCACGATCAGCAGCATGGAGGCTGAAGATGCTGCCACTT 250
SerTyrSerLeuThrIleSerSerMetGluAlaGluAspAlaAlaThrTyr

ATTATTGTTATCAGGGGAGTGGGTACCCATTCACGTTCGGCTCGGGGACA 300
TyrCysTyrGlnGlySerGlyTyrProPheThrPheGlySerGlyThr

AAGTTGGAAATAAAAGGTTCTACCTCCGGATCTGGTAAATCTTCTGAAGG 350
LysLeuGluIleLysGlySerThrSerGlySerGlyLysSerSerGluGly

TAAAGGTGTGCAGCTGCAGCAGTCTGGGGTTGAGCTTGTCCGAGGAGGGG 400
LysGlyValGlnLeuGlnGlnSerGlyValGluLeuValArgGlyGlyAla

CCTTAGTCAAGTTGTCCTGCAAAGCTTCTGACTTCAACATTAAAGACTAT 450
LeuValLysLeuSerCysLysAlaSerAspPheAsnIleLysAspTyr

TATATCCACTGGGTGAAGCAGAGGCCTGAACAGGGCCTGGAATGGATTGG 500
TyrIleHisTrpValLysGlnArgProGluGlnGlyLeuGluTrpIleGly

ATGGATTCATCCTGAGAATGGTAATACTGTATATGACCCGAAATTCCAGG 550
TrpIleHisProGluAsnGlyAsnThrValTyrAspProLysPheGlnGly

GCAAGGCCAGTATAACAGCAGACACATCCTCCAACGCGGCCTACCTTCAG 600
LysAlaSerIleThrAlaAspThrSerSerAsnAlaAlaTyrLeuGln

CTCAGCAGCCTGACATCTGAGGACACTGCCGTCTATTACTGTGCTTCTTA 650
LeuSerSerLeuThrSerGluAspThrAlaValTyrTyrCysAlaSerTyr

```
TTACTACTATAGTGCTTACTATGCTATGTACTACTGGGGTCAAGGAACCT   700
 TyrTyrTyrSerAlaTyrTyrAlaMetTyrTyrTrpGlyGlnGlyThrSer

CGGTCACCGTCTCCTCATAA  720
  ValThrValSerSerTer
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08545

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 35/14, 39/00; C12N 15/00; C07K 3/00, 13/00, 15/00, 17/00
US CL :424/85.8; 435/172.2; 530/388.22, 388.8, 389.7
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 435/172.2; 530/388.22, 388.8, 389.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG and APS
search terms: erbB-2, antibody and malignant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
L	Science, Vol. 252, issued 21 June 1991, Waldmann "Monoclonal Antibodies in Diagnosis and Therapy", pages 1657-1661, see entire document (provided to question the method of treatment claims, since use of monoclonal antibodies in vivo is taught to be unpredictable).	5-11
Y	Cancer Research, Vol. 50, issued 01 March 1990, Fendly et al., "Characterization of Murine Monoclonal Antibodies Reactive to Either the Human Epidermal Growth Factor Receptor or HER2/neu Gene Product", pages 1550-1558, see entire document.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 APRIL 1993	17 MAY 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DONALD E. ADAMS, PH.D. Telephone No. (703) 308-0196

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